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Cladal relatedness among *Aspergillus oryzae* isolates and *Aspergillus flavus* S and L morphotype isolates

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Abstract

Aspergillus flavus is the main etiological agent for aflatoxin contamination of crops. Its close relative, *A. oryzae*, does not produce aflatoxins and has been widely used to produce fermented foods. We compared the phylogeny of *A. oryzae* isolates and L- and S-type sclerotial isolates of *A. flavus* using single nucleotide polymorphisms in the *omtA* gene in the aflatoxin biosynthesis gene cluster and deletions in and distal to the *norB-cypA* intergenic region as phylogenetic signals. Aflatoxin-producing ability and sclerotial size also were weighted in the analysis. Like *A. flavus*, the *A. oryzae* isolates form a polyphyletic assemblage. *A. oryzae* isolates in one clade strikingly resemble an *A. flavus* subgroup of atoxigenic L-type isolates. All toxigenic S-type isolates closely resemble another subgroup of atoxigenic L-type isolates. Because atoxigenic S-type isolates are extremely rare, we hypothesize that loss of aflatoxin production in S-type isolates may occur concomitantly with a change to L-type sclerotia. All toxigenic L-type isolates, unlike *A. oryzae*, have a 1.0 kb deletion in the *norB-cypA* region. Although *A. oryzae* isolates, like S-type, have a 1.5 kb deletion in the *norB-cypA* region, none were cladally related to S-type *A. flavus* isolates. Our results show that *A. flavus* populations are genetically diverse. *A. oryzae* isolates may descend from certain atoxigenic L-type *A. flavus* isolates.
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Keywords: *Aspergillus oryzae*; *Aspergillus flavus*; Phylogenetics; Sclerotia; Aflatoxin gene cluster

1. Introduction

Aspergillus flavus populations are genetically diverse and phenotypic variations have been well documented (Cotty, 1989; Geiser et al., 2000; Horn and Dorner, 1999; Pildain et al., 2004; Takahashi et al., 2004). *A. flavus* isolates vary considerably in their abilities to produce aflatoxins and colonize plants (Mellon and Cotty, 2004). They generally can be grouped into two sclerotial morphotypes, L strain and S strain (also named *A. flavus* var. *parvisclerotigenus* (Saito and Tsuruta, 1993). L strain isolates produce abundant conidiospores and sclerotia that are usually larger than 400 μm in diameter (Cotty, 1989; Horn and Dorner, 1999), whereas S strain isolates produce fewer conidiospores and numerous sclerotia that are usually smaller than 400 μm in diameter. The S strain isolates typically produce higher amounts of aflatoxin than the L strain isolates on the same media (Bayman and Cotty, 1993; Novas and Cabral, 2002). The aflatoxigenic trait of the S strain isolates seems very stable. In contrast, a significant portion of *A. flavus* L strain field isolates do not produce aflatoxins (Horn and Dorner, 1999; Mphande et al., 2004; Pildain et al., 2004; Tran-Dinh et al., 1999; Vaamonde et al., 2003). Despite this dichotomy, the genetic relationship between L strain and S strain is still not understood. The divergence of L and S strains has been estimated to occur between 1 and 3 million years ago (Ehrlich et al., 2005).

Aspergillus oryzae is a morphologically similar, nonaflatoxigenic relative of *A. flavus* and generally produces abundant conidiospores, but no or few sclerotia. Isolates of *A. oryzae* have been used as a source of many important

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industrial enzymes and as a koji (starter) mold for Asian fermented foods, such as sake, miso, and soy sauce (van den Broek et al., 2001). On the basis of DNA complementarity, Kurtzman et al. (1986) found that *A. oryzae* is closely related to *A. flavus*. Geiser et al. (1998) suggested that *A. oryzae* isolates arise from domestication of *A. flavus* subgroup isolates. Because *A. oryzae* has GRAS (generally regarded as safe) status for use in the food industry, efforts have been made to develop molecular methods to unambiguously distinguish *A. oryzae* from *A. flavus*. These methods include restriction fragment length polymorphism (Klich and Mullaney, 1987), amplified fragment length polymorphism (Montiel et al., 2003), electrophoretic karyotyping (Keller et al., 1992), isozyme profiling (Cruickshank and Pitt, 1990), and analysis of ribosomal DNA internal transcribed spacer regions (Kumeda and Asao, 2001). Generally, these methods have not been successful in unambiguously separating *A. oryzae* as a distinct species.

In the present study, we compare single nucleotide polymorphisms in the gene, *omtA*, and DNA sequence deletions in isolates of *A. oryzae*, toxigenic S-morphotype *A. flavus* and both toxigenic and atoxigenic L-morphotype *A. flavus*. From this comparison we found that *A. oryzae* isolates belong to specific subgroups of atoxigenic L-type isolates. Our results suggest that sclerotial morphotype is a poor indicator of phylogenetic relationships among *A. flavus* isolates.

2. Materials and methods

2.1. Fungal isolates

Isolates of *A. oryzae* used in this study are SRRC304 (NRRL1808, ATCC1808; isolated from moldy bran, USA), SRRC493 (NRRL3485; isolated from miso, Taiwan), SRRC2044 (FRR2974; isolated from maize, New Zealand), SRRC2098 (FRR1677, NRRL2217, ATCC11493, IMI52144; isolated from soybean–wheat flour mixture, Japan), SRRC2103 (ATCC10196; isolated from pine panel, USA), and RIB40 (ATCC 42149; isolated from cereal, Japan). RIB40 is the strain used for EST (expressed sequence tag) and genome sequencing projects by the *A. oryzae* genome sequencing consortium of Japan. Isolates of *A. flavus*, which belong to different sclerotial morphotypes and either produce or do not produce aflatoxins are summarized in Fig. 1. *A. flavus* AF12 and AF13 were collected from Arizona cotton fields (Cotty, 1989). The CA designated isolates were collected from pistachio buds in the Wolfskill Grant Experimental Farm (University of Davis, Winters, California). Other *A. flavus* isolates with capital letter abbreviations indicating the state from which an isolate was collected were obtained from National Peanut Research Laboratory (Dawson, Georgia). They were collected from agricultural soils of southern United States (Horn and Dorner, 1998, 1999). *A. flavus* NRRL3357 is the strain currently used for *A. flavus* genome sequencing project by Payne et al. at North Carolina State University (Raleigh, North Carolina). Abbreviations for the culture collection organizations are as follows: ATCC — American Type Culture Collection; NRRL — Northern Regional Research Laboratory (now called National Center for Agricultural Utilization Research); SRRC — Southern Regional Research Center; FRR — Food Science Australia; IMI — CAB International Mycological Institute; RIB — National Research Institute of Brewing.

2.2. DNA extraction

Adye and Mateles (1964) medium was used to grow submerged cultures for preparation of genomic DNA. Frozen mycelia was ground to a fine powder in liquid nitrogen and DNA was isolated using a GenElute™ Plant Genomic DNA Miniprep kit (Sigma, St. Louis, Missouri, USA).

2.3. Determination of deletions in and distal to the aflatoxin gene cluster in A. flavus and A. oryzae isolates

Oligonucleotide primers for PCR were derived from the aflatoxin gene cluster and the distal sequence flanking the cluster of *A. flavus* (AY510451) or *A. parasiticus* (AY371490). The norB-cypA primer set, 5′-GTGCCCAGCATCTTGGTCCA-3′ and 5′-AGGACTTGATGATTCCTCGTC-3′, was used to amply unique deletion regions found in *A. flavus* (Ehrlich et al., 2004). Other primers sequences are C1: 5′-CGTTCCAGTAGTTCGTATCG-3′ and 5′-CATCGTAAACGTTGACACAG-3′; C2: 5′-TCGCCTTGTTCTCGCTATAC-3′ and 5′-ACACCTGATAGCGAGAGTTC-3′; C3: 5′-GCGATCTGTAACACTACACA-3′ and 5′-GCCATACGATTCCCAAGTCT-3′; and omtA: 5′-CAGGATATCATTGTGGACGG-3′ and 5′-CTCCTCTACCAGTGGCTTCG-3′. The sequences of other paired aflatoxin biosynthetic gene primers are detailed in a previous study (Chang et al., 2005). PCR screenings for deletions in the aflatoxin gene cluster and a flanking region were performed under the following conditions in a Perkin Elmer GeneAmp PCR System 2400. Primers and genomic DNA were added to 50 μL Platinum® Blue PCR Supermix (Invitrogen, Carlsbad, California, USA) and heated at 94 °C for 5 min and then subjected to 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1.5 min. A final 7-min extension step at 72 °C was included. All PCR products obtained by the norB-cypA primers were sequenced at Iowa State University DNA Sequencing and Synthesis Facility (Ames, Iowa, USA).

2.4. Determination of the junction of the sequence breakpoints in the aflatoxin gene cluster in A. oryzae isolates

The deletion patterns determined by PCR in the aflatoxin gene cluster in *A. oryzae* SRRC2098 and SRRC2103 resemble the pattern E deletion found in some *A. flavus* isolates (Chang et al., 2005). Two primers, PE47: CCATCGCATCAGCATTCT and V70R: CGCCGTCGCCTCAGGATCC, derived from regions flanking the junction of the sequence breakpoints in *A. flavus* TX13-5 (AY987855) were used to amplify the region that putatively contained the same breakpoints. The GenBank sequence accession numbers for the sequences in isolates

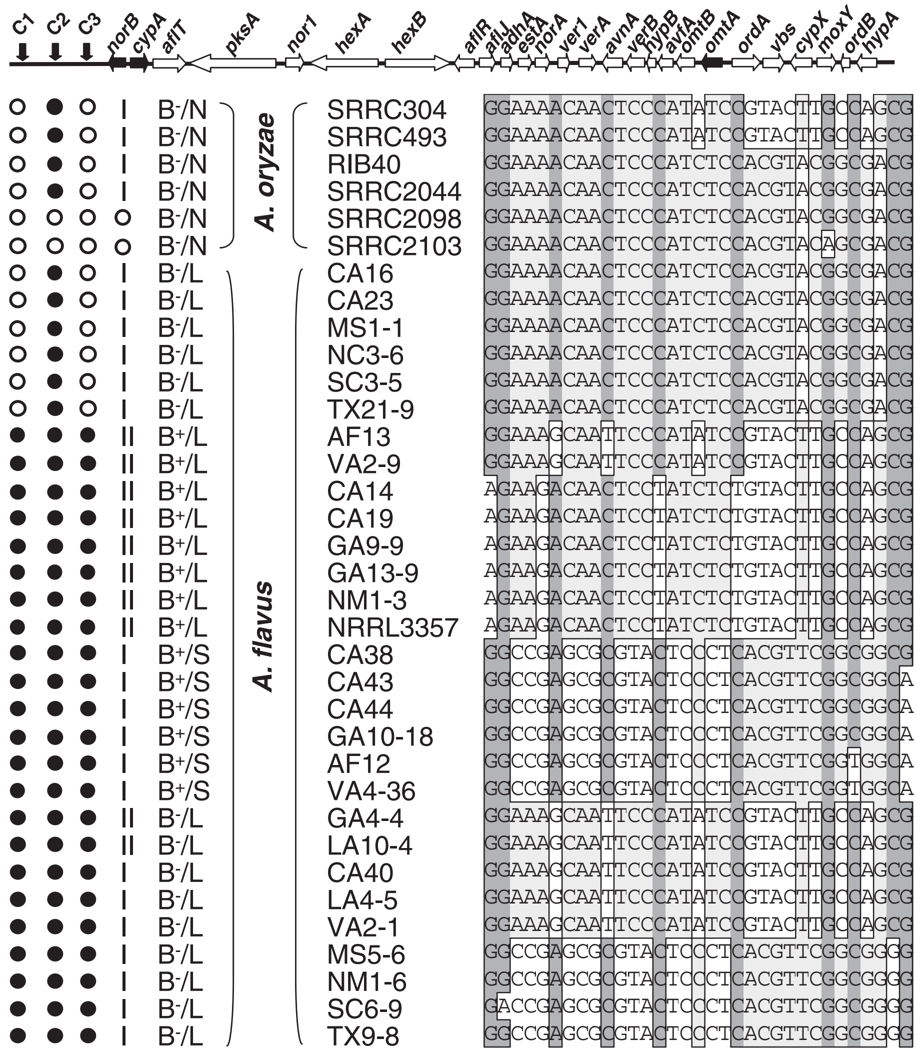

Fig. 1. Deletions in and distal to the *norB-cypA* region in the aflatoxin gene cluster and polymorphisms in *omtA* of *A. oryzae* and *A. flavus* isolates. The top part of the figure is a schematic depiction of the aflatoxin biosynthesis gene cluster. Solid arrows indicate the regions examined in this study. Solid circles indicate positive PCR products and open circles indicate no PCR products. I: Type I deletion in the *norB-cypA* region. II: Type II deletion in the *norB-cypA* region. C1, C2, and C3 are small regions distal to the aflatoxin gene cluster toward the telomere. N: no or immature (nonmelanized) sclerotia. L: large sclerotia; S: small sclerotia. B$^+$: aflatoxigenic; B$^-$: nonaflatoxigenic. See Materials and methods for strain designations. The right panel is the comparison of the compiled SNPs in the *omtA* gene among *A. oryzae* and *A. flavus* isolates.

SRRC2098 and SRRC2103 are DQ112071 and DQ112070, respectively.

2.5. Single nucleotide polymorphisms in the omtA gene of A. oryzae and A. flavus isolates

A portion of the *omtA* gene of *A. flavus* and *A. oryzae* was PCR amplified and directly sequenced. This 594 bp region, which is larger than the 420 bp region analyzed by Geiser et al. (2000) contains introns I, II, and III and coding regions for amino acids 97 to 237 of OmtA. The single nucleotide polymorphisms (SNPs) are present at positions of 340, 343, 354, 357, 375, 376, 377, 378, 385, 387, 392, 394, 400, 421, 427, 428, 453, 465, 468, 486, 501, 517, 525, 531, 546, 583, 624, 637, 649, 651, 755, 756 and 766 where position 1 is A in the *A*TG start codon. Sixteen of these sites are in the introns and 17 are in coding regions.

2.6. Phylogenetic analysis

The phylogenetic dataset included the above *omtA* SNPs and characteristic deletions distal to and within the aflatoxin gene cluster (Fig. 1). The deletions distal to the aflatoxin gene cluster, the deletion in the *norB-cypA* region, the ability to produce aflatoxins, and the sclerotial type were arbitrarily assigned twice the weight of single nucleotide changes because such deletions, chemotype, and morphotype likely arise from complicated genetic changes. A total of 42 characters were used in the analysis. Two parsimony uninformative sites of the *omtA* SNPs were removed during the sampling. Phylogenetic analysis of the alignment dataset used the Distance (Minimal Evolution) method performed with PAUP*, version 4.0b10 (Sinauer Associates, Sunderland, MA), based on heuristic search routine. Bootstrap support values are based on 1000 replicates. The outgroup for the analyses was *A. parasiticus* SU-1.

3. Results and discussion

In this study we compared the sequences of DNA regions both in and distal to the aflatoxin cluster (Fig. 1) in order to deduce the phylogenetic relationships among isolates of *A. oryzae* and aflatoxin-producing and aflatoxin-nonproducing *A. flavus*. A comparison of six *A. oryzae* isolates and 27 *A. flavus* isolates indicates that 33 nucleotide sites in the *omtA* gene region are polymorphic (Fig. 1). The *omtA* gene in the aflatoxin gene cluster was previously used in the phylogenetic studies of *A. flavus* and *A. oryzae* isolates (Geiser et al., 1998, 2000). The sequence was also used to distinguish the two species (van den Broek et al., 2001). The work of Geiser et al. (2000) supported the separation of *A. flavus* isolates into at least three distinct clades. Compared to other genes in the cluster, *omtA* provided a greater number of phylogenetically informative sites (Ehrlich et al., 2005; unpublished results).

Besides SNPs in the *omtA* gene we included, as significant phylogenetic signals, characteristic deletions at the distal end of the aflatoxin gene cluster (e.g., the *norB-cypA* region) and in the region between the gene cluster and the telomere (Fig. 1). All typical *A. flavus* isolates produce only B aflatoxins. This is because of a deletion in the *norB-cypA* intergenic region that prevents the biosynthesis of a cytochrome P450 monooxygenase required for G aflatoxin formation (Ehrlich et al., 2004). It is possible that this region is more prone to deletions because it is at the distal end of the aflatoxin cluster and resides near the subtelomeric region of the chromosome (Chang et al., 2005). It is known that subtelomeric regions have a particularly high rate of genetic flux when compared to other chromosomal regions (Liti and Louis, 2005; Maciaszczyk et al., 2004). Two types of deletions (Type I, 1.5 kb and Type II, 1.0 kb) are found in the *norB-cypA* regions of *A. flavus* isolates when this sequence is compared to the sequence in species of *Aspergillus* that produce both B and G aflatoxins. Type I deletion in the *norB-cypA* region overlaps mostly with type II deletion. However, Type II contains a 32 bp deletion in the region encoding amino acid residues 300–310 of the predicted NorB protein that is not found in Type I. Thus, Type I deletion and Type II deletion arise from independent DNA loss in the *norB-cypA* region.

The current study includes a greater number of atoxigenic *A. flavus* and *A. oryzae* isolates than previous studies (Geiser et al., 1998, 2000). Our phylogenetic analysis (Fig. 2) shows that relationships among *A. flavus* isolates are similar to those previously found by Geiser et al. (2000). Like their study, we find that *A. flavus* is a polyphyletic assemblage of isolates, and although they found *A. oryzae* isolates to be monophyletic, we find that *A. oryzae,* like *A. flavus*, is polyphyletic (Fig. 1). The *A. oryzae* isolates separate into three distinct clades that are nested in clades that contain atoxigenic L-type *A. flavus* isolates. Four of the *A. oryzae* isolates (RIB40, SRRC2044, SRRC2098, and SRRC2103) are in a clade that only contains *A. flavus* isolates with Type I deletion in the *norB-cypA* region and distinct sequence variations distal to the aflatoxin gene cluster (Fig. 2). Based on the *omtA* SNPs, SRRC2098 and SRRC2103 probably descend from the same progenitor as RIB40 and SRRC2044 but have lost a major part of the aflatoxin gene cluster (Fig. 1). The deletion in SRRC2098 and SRRC2103 extends from 300 nt proximal to the start codon of the *ver1* gene (Fig. 3) to a region beyond the end of the *norB* gene that contains a gene encoding a predicted protein that has 52% identity to *A. nidulans* AmdA (Lints et al., 1995). Kusumoto et al. (2000) reported a similar group of *A. oryzae* isolates, which have a large deletion distal to the *ver1* gene. The *A. oryzae* isolates SRRC2044, RIB40, SRRC304, and SRRC493, like *A. oryzae* group I isolates classified by Kusumoto et al. (2000), contain a complete aflatoxin gene cluster based on PCR assays with aflatoxin gene-specific primers (data not shown), but they are cladally distinct. Considering the relatively short history of human use of *A. oryzae*, progenitors of *A. oryzae* likely are atoxigenic L-type *A. flavus*, isolates of which are still found in many part of the United States.

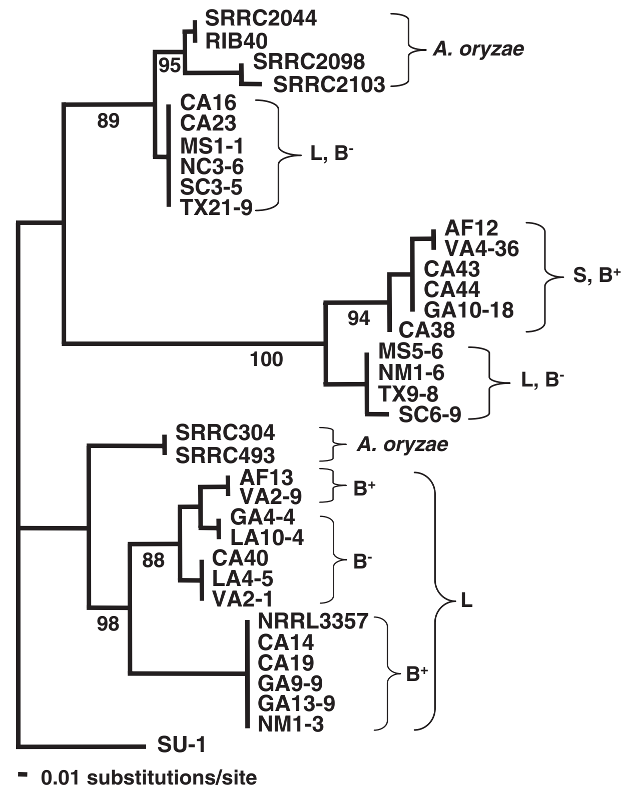

Fig. 2. Phylogenetic analysis of *A. oryzae* and *A. flavus* isolates. Analysis was performed by Distance criterion (Minimal Evolution, ME) in PAUP using heuristic search methods with neighbor joining. Of 42 characters 40 were parsimony informative. The 50% majority rule consensus tree is shown. The ME score is 1.41422. Bootstrap support values based on 1000 replicates are shown under the branches. *A. parasiticus* was used as the outgroup.

The clade containing toxigenic S-type isolates (AF12, VA36, CA43, CA44, GA10-18, and CA38) includes atoxigenic L-type isolates (MS5-6, NM1-6, TX9-8, and SC6-9) (Fig. 2). These L-type isolates also have the S-type (Type I) deletion in the *norB-cypA* region (Fig. 2). To explain this result we suggest that loss of aflatoxin production in S-type isolates occurs concomitantly with a change in sclerotial morphology. This offers an explanation of the observation that natural *A. flavus* isolates that produce small sclerotia are rarely atoxigenic, if ever found

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TCTACCACGCCGTCCTGCAATCCATTCACGCGCTTTCCATTCCCGTTCGTATCGGCGTG
GAATACGTCGCCAGAACGCAGACCTTGACATGGAGCACGATCCATTCGCTCTGTAACCT
CGAATGCGCCCTCTTCCTATGTAAATGGCTCGACACGTTCGCGTCgggcccggcatttc
ttcattgagacacacggcg▲gttttcgtttattattgtgtttttggtgtgattggttta
gagcctgctcctattctcagcttcctatgctttcagcctaacataaacaagacgtatta
ctacacagaagttttgggctcgccgcccgatgagctacaggtattcagatatttcggtc
tccgaggaaagatttgtttggtggccaaccatccatagctgcgtatatatgtagtgcat
gaccggtcccatggatcaccgttttaacagaactacacatcattttgcctccctaaagt
ctctaccccagacgatttcttcaacATGTCCGACAACCACCGTTTAGATGGCAAAGTGG
                          (Ver1)M  S  D  N  H  R  L  D  G  K  V
CCTTAGTCACCGGCGCCGGCCGCGGCATCGGTGCTGCCATCGCCGTCGCGCTTGGTGAG
A  L  V  T  G  A  G  R  G  I  G  A  A  I  A  V  A  L  G  E
CGCGGAGCCAAAGTCGTGGTGAACTACGCCCATTCCCGTGAGGCCGCGGAGAAAGTGGT
 R  G  A  K  V  V  V  N  Y  A  H  S  R  E  A  A  E  K  V  V
TGAACAGATCAAGGCCAATGGTACCGACGCTATCGCAATCCAGGCCGATGTCGGG
  E  Q  I  K  A  N  G  T  D  A  I  A  I  Q  A  D  V  G
```

Fig. 3. Sequence of the breakpoint junction around the *amdA* and *ver1* genes in *A. oryzae* SRRC2044 and SRRC2103. The portion highly homologous to *A. nidulans amdA* is underlined and in upper case. A solid triangle indicates the junction of two sequence breakpoints. The coding sequence is in upper case and the noncoding sequence is in lower case.

(Cotty, unpublished results). Other studies have provided evidence that sclerotial development and aflatoxin production are related processes (Calvo et al., 2004; Cotty, 1988). Consistent with this idea is a previous finding that mutants producing elevated levels of aflatoxin intermediates by forced-expression of the aflatoxin pathway-specific regulatory gene *aflR* yield reduced sizes of sclerotia (Chang et al., 2001). In a different study, Garber and Cotty (1997) found that an atoxigenic L-type *A. flavus* isolate used as a bio-competitor to reduce aflatoxin contamination of cottonseed is more effective at reducing contamination by S-type isolates than L-type isolates. This isolate, AF36, was found to have the S-type deletion in the *norB-cypA* region and characteristic S-type SNPs in its *omtA* gene (results not shown). Marked competition likely occurs between genetically similar *A. flavus* isolates regardless of sclerotial morphotypes. It was previously found that the toxigenic S-type *A. flavus* isolates grouped phylogenetically with or within clades of certain atoxigenic L *A. flavus* isolates (Egel et al., 1994; Geiser et al., 2000).

All of the aflatoxin-producing L-type isolates have the Type II deletion in the *norB-cypA* region. Two atoxigenic L-type isolates (GA4-4 and LA 10-4) also have this type of deletion, but all other atoxigenic L-type isolates have the Type I deletion (Fig. 2). GA4-4 and LA10-4 most likely derive from a similar toxigenic L-type ancestor since, in all respects other than aflatoxin production, they resemble Type II isolates. Generally, aflatoxin-producing ability seems to be relatively stable in L-type isolates with the Type II *norB-cypA* deletion while Type I deletion is associated with both toxigenic S-type *A. flavus* isolates, *A. oryzae* isolates, and most atoxigenic L-type *A. flavus* isolates (Fig. 1). None of the *A. oryzae* isolates are cladally related to toxigenic S-type *A. flavus* isolates. Some atoxigenic *A. flavus* isolates (CA40, LA4-5, and VA2-1) contain the Type I *norB-cypA* deletion but have an *omtA* sequence that is almost identical to atoxigenic L-type *A. flavus* isolates (GA4-4 and LA10-4) that contain the Type II deletion. The mixed pattern could arise by genetic recombination as previously proposed (Geiser et al., 1998; Tran-Dinh et al., 1999). These isolates may share a, as yet unrecognized, common ancestor from which another clade of *A. oryzae* isolates (SRRC304 and SRRC493) descends.

By including in the phylogenetic analysis morphological and biosynthesis characteristics with *omtA* SNPs and sequence deletions, we have been able to differentiate *A. flavus* and *A. oryzae* isolates into multiple, well-supported clades. This analysis provides a detailed picture of the relatedness among *A. flavus* and *A. oryzae* subgroups. The unique *omtA* SNPs and distinct deletions in the region distal to the *norB* gene (see Fig. 1, C1 and C3) would provide a basis for the selection of nonaflatoxigenic *A. flavus/oryzae* isolates for use in industrial fermentation. At present, we do not know if the *norB-cypA* region is a mutational hot-spot for the characteristic deletions found in *A. flavus* and *A. oryzae*. Information from the completed *A. oryzae* RIB40 (Type I) genome sequence and the ongoing *A. flavus* NRRL3357 (Type II) genome sequence project may shed yet more light on the evolutionary origins and presumed genetic relatedness of these *A. flavus* subspecies.

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